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1. Waters Clin Lab Haematol. (1999) <sup>21</sup>12(3): 169-172
2. Howard J. anat. (1996) 189(Pt.2 ): 303-313
3. Wahlstedt Br. J. Haematol (1990) 76(3): 420-426
4. Vaillant Cell Tissue Res. (1990) 260(1): 117-122
5. Levine J. Cell Biol. (1981) 90(3): 644-655
6. McKay Res. Vet Sci (1981) 30(3): 261-265
7. Levine Gastroenterology (1980) 79(3): 493-502

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## An enzyme immunoassay for intrinsic factor in urine

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**Summary** An enzyme immunoassay for intrinsic factor has been used on urine. The assay can measure intrinsic factor in native urine from healthy people and from patients with pernicious anaemia with no antibodies. The urinary intrinsic factor concentration in healthy individuals ranged from 40 to 54 pmol/l. Intrinsic factor antibodies, demonstrated by testing the recovery of added intrinsic factor, interfered with the assay. Cobalamin at high concentrations also affected the assay result. A low intrinsic factor concentration or the presence of antibodies to intrinsic factor was found in the urine of individuals with pernicious anaemia.

**Keywords** Cobalamin, enzyme immunoassay, intrinsic factor, urine

### Introduction

A substance in normal urine that cross-reacts immunologically with intrinsic factor (IF) has been detected by radioimmunoassay (Grasbeck, Wahlstedt & Kouvonon 1982). The assay promised a noninvasive test in the diagnosis of pernicious anaemia but the subsequent demonstration that IF antibodies (IFAB) interfered with the assay (Wahlstedt 1990), together with the need for a laborious urine concentration step (Grasbeck *et al.* 1982), limited the value of the procedure. The estimation of IF in urine, if shown to identify pernicious anaemia, would avoid the need for a Schilling test in many individuals. A sensitive enzyme immunoassay for IF (Waters 1996) has therefore been used to measure IF in native urine. We describe our findings regarding the assay of IF in urine and the effect of IFAB on the test.

### Materials and methods

IF and IFAB preparations were made as described previously (Waters *et al.* 1989; Waters 1996). As one molecule of cobalamin is bound by one molecule of IF, and although the weight of cobalamin in an IF/cobalamin complex will be constant, that of the IF may vary (Hall 1983), and consequently molar equivalent units have been used to express the quantity of IF. IFAB units are expressed as the inhibition of IF–cobalamin binding. One nanogram unit (ngu) of IFAB is defined as that amount inhibiting the binding of 1 ng of cobalamin (Chanarin 1979).

The samples tested were:

1) Urine samples from 10 healthy individuals (these, and other samples from healthy individuals, were termed controls).

2) Urine from an individual who had undergone a total gastrectomy.

3) Urine from a healthy person, untreated and with added 74 and 148 pmol/l IF, for precision studies (each sample was assayed 8 times on six occasions).

4) Urine from a healthy person, with an IF concentration of 50 pmol/l, seeded with IF to give increments of 37, 74 and 148 pmol/l for recovery studies (the 10 control urine samples listed above were also assayed with the addition of 370 pmol/l IF and recovery was taken to be reduced when < 66% of added IF was detected).

5) IFAB in concentrations ranging from 0.08 to 10.0 µgU/l added to a control urine sample seeded with 370 pmol/l IF to test the effect of antibody on the assay system.

6) A control urine sample with cobalamin in doubling dilutions added to give eight concentrations from 1000 to 16 ng/l and another eight from 1000 to 8 µg/l to test the effect of cobalamin on the system (the urine samples were assayed with and without the addition of 370 pmol/l IF).

7) Thirteen random urine samples from patients with pernicious anaemia, not collected during a Schilling test, unseeded and seeded with 74 and 370 pmol/l IF added. The urinary cobalamin levels were all < 500 ng/l. Cobalamin assays were carried out using a radioisotope dilution assay (Matthews, Gunasegeram & Linnell 1967). The diagnosis of pernicious anaemia was based upon a low serum cobalamin, IFABs in the serum or aqueous cobalamin absorption tests, and the response to cobalamin therapy.

## Results

### Intrinsic factor assay

The 10 control urine samples gave a mean IF concentration of 47.4 pmol/l with a range of 40–54 pmol/l. Their cobalamin content ranged from 33 to 103 ng/l.

The IF concentration in the urine, cobalamin content 207 ng/l, from the patient who had undergone a total gastrectomy was < 8 pmol/l.

### Precision

The control urine samples without and with added IF gave the following results: within-batch; at an IF concentration of 42 pmol/l the CV range was 9.5–20.6%, at 104 pmol/l the range was 6.3–11.7% and at 159 pmol/l the range was 6.3–14.6%. Between-batch CV ranges at the same concentrations were 5.5–18.7%, 10.7–20.0% and 4.6–18.4%, respectively.

### Recovery

The mean recovery from a control urine sample, of 37 pmol/l IF was 94%, of 74 pmol/l IF was 103% and of 148 pmol/l IF was 136%.

Ten control urine samples, with 370 pmol/l IF added, gave a mean recovery of 89% (range 67–117%).

### Effect of IFAB on the IF assay

With antibody levels of 0.63 µgU/l, recovery of 500 pmol/l IF was significantly reduced. Higher IFAB concentrations reduced the IF level to below the control range (Table 1).

### Effect of cobalamin on assay and on recovery of IF

Addition of cobalamin up to a concentration of 1.0 µg/l did not affect the assay of IF (mean without cobalamin 61 pmol/l, with cobalamin 57 pmol/l) or the recovery of

370 pmol/l IF (mean without cobalamin 92%, with cobalamin 88%).

The assay of IF in control urine samples was not affected by cobalamin concentrations from 8 to 1000 µg/l (mean without added cobalamin 55 pmol/l, with 53 pmol/l). The mean recovery of 370 pmol/l IF was 103%. However, with these higher concentrations of cobalamin, recovery of this level of IF was reduced and showed an inverse relationship to the cobalamin content (Table 2).

### Assay of urinary IF and recovery of added IF in pernicious anaemia

The assay of the 13 random urine samples from individuals with pernicious anaemia, with normal or slightly raised cobalamin content (Table 3), showed 10 with a low and three with an apparently normal IF content. The recovery of added IF was < 66% in six (of 370 pmol/l IF from three, and of 74 pmol/l IF from five). Recovery was unrelated to the cobalamin level.

The result of the IF assay in these six was considered to be invalid because of a poor recovery of added IF. This applied to three with a low IF content and to three (numbers 2, 12 and 13) with an apparently normal IF content.

## Discussion

We have modified our IFAB enzyme-linked immunosorbent assay (ELISA) to produce a competitive binding enzyme immunoassay for the measurement of IF in urine. The assay results show that this method is of a sufficient sensitivity to detect the small amounts of IF that are found to be present in normal urine by radioimmunoassay (Grasbeck *et al.* 1982). The high CVs were probably associated with the very low levels of IF. Two factors, the presence of IFAB and of cobalamin, were found to interfere with the test.

**Table 1.** Effect of intrinsic factor antibodies (IFAB) on recovery of intrinsic factor (IF) from urine

	IFAB concentration (µgU/l)									
	0	0.08	0.16	0.31	0.63	1.25	2.50	5.0	10.0	
IF (pmol/l)	466	518	444	488	289	37	<10	<10	<10	
recovery (%)		111	95	105	62	8	<2	<2	<2	

**Table 2.** Effect of cobalamin on recovery of intrinsic factor (IF)

Cobalamin added (µg/l)	Recovery of 370 pmol/l IF (%)	
	Urine	Urine + cobalamin
1000	125	7
500	103	7
250	81	11
125	81	15
62.5	85	16
31.2	81	13
15.6	119	31
7.8	145	48

**Table 3.** Assay of urinary intrinsic factor (IF) and of added IF in pernicious anaemia

Patient	Cobalamin (ng/l)	IF assay (pmol/l)	Recovery of added IF (%)		Serum IFAB
			74 pmol/l	370 pmol/l	
1	59	17	102	101	NA
2	48	48	35	79	+
3	453	10	30	87	+
4	70	16	86	94	-
5	52	12	89	99	-
6	40	10	103	134	-
7	67	16	124	116	+
8	27	37	85	134	-
9	52	29	91	36	+
10	<20	30	59	96	+
11	<20	38	113	66	-
12	74	92	15	61	-
13	293	46	48	33	+
Control	288	48	98	99	

NA, not available. +, positive. -, negative. IFAB, intrinsic factor antibody.

Wahlstedt (1990) demonstrated the presence of both type I and type II IFAB in urine which gave false positive results for IF and considered that the radioimmunoassay of IF cannot be used to diagnose pernicious anaemia until this antibody interference is eliminated. Our tests showed that IFABs of  $\geq 0.63 \mu\text{gU/l}$  inhibited the recovery of 370 pmol/l IF and that the recovery of  $\leq 74 \text{ pmol/l}$  IF was inhibited by lower levels of IFAB but 'enhanced', because of a hook effect, by concentrations  $> 0.63 \mu\text{gU/l}$  (Waters 1996). We therefore included the recovery of 74 pmol/l IF in an attempt to detect a wider range of IFAB concentrations. We agree with Wahlstedt (1990) that the presence of IFABs may negate the assay of IF in urine. However, by including IF recovery tests with the assay we believe that their detection can be used as a positive step toward the diagnosis. It is generally accepted that, in a patient suspected of having pernicious anaemia, IFABs in the plasma are indicative of the diagnosis and this assumption is likely to apply to their presence in other body fluids. Discrepancy between the incidence of IFAB in the urine and serum is not surprising. The sensitivity of the two assays differ and Wahlstedt (1990) recorded differing IFAB concentrations and types in urine and serum. Using gel filtration techniques antibodies were detected in the sera of four patients, with antibodies in the urine of three. We have not tested patients with other autoimmune diseases or with cobalamin deficiency as a result of other causes.

Cobalamin did not appear to interfere with the assay of IF in control urine samples but when present in Schilling test amounts did interfere with the recovery of higher concentrations. This reduction in recovery, inversely related to the level of cobalamin present, was probably associated with the solid phase support. At low concentrations of IF in the sample most, whether complexed with cobalamin or not, would be taken up preferentially by any type II IFAB in the preparation and therefore even high concentrations of cobalamin will have little or no effect on competition between labelled and unlabelled IF. However, with higher concentrations of IF complexed to cobalamin, proportionally less will be captured by the solid phase compared with free IF since the former will only react with type II IFAB and the latter is more likely to be taken up by both type I and type II antibodies. At equilibrium a higher proportion of label would therefore be associated with the solid phase in the case of complexed IF indicating a lower than expected concentration and therefore reduced recovery.

The very low level of IF in the urine of the patient who had undergone a total gastrectomy supports the suggestion that this IF originates in the stomach (Grasbeck *et al.* 1982). A gastric source of urinary IF may give a postprandial surge which would affect the reference range. Our control samples were provided by laboratory staff in the mid-morning and the pernicious anaemia urine samples, other than those associated with Schilling tests, were collected during a morning clinic. Standardization of the time is desirable. Renal failure may also affect the result.

The assay is capable of estimating the level of IF in native urine. Providing that the level of cobalamin is  $\leq 1.0 \mu\text{g/l}$  in the assay, and an IF recovery test is used, the assay will detect the presence of IFAB or, if this is absent, assay IF. Pernicious anaemia was the probable diagnosis, based upon both the IF and the recovery tests, in all 13 patients, although the results in patients 8 and 11 were equivocal. Seven of the patients had previously required a Schilling test for diagnosis. Had both urine results been available, only these two patients (8 and 11) would have needed this procedure.

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### References

- Chanarin I. (1979) *The Megaloblastic Anaemias*, 2nd edn. Blackwell Scientific Publications, Oxford.

- Grasbeck R., Wahlstedt V. & Kouvonon I. (1982) Radioimmunoassay of urinary intrinsic factor. *Lancet* **I**, 1330-1332.
- Hall C.A. (1983) The transport of cobalamin - Introduction. In: Hall C.A., ed. *Methods in Haematology, Volume 10: The Cobalamins*. Churchill-Livingstone, Edinburgh, pp. 75-77.
- Matthews D.M., Gunasegaram R. & Linnell J.C. (1967) Results with radioisotopic assay of serum B<sub>12</sub> using serum binding agent. *Journal of Clinical Pathology* **20**, 683-686.
- Wahlstedt V. (1990) Urinary autoantibodies against intrinsic factor in pernicious anaemia. *British Journal of Haematology* **76**, 420-426.
- Waters H.M. (1996) Vitamin B<sub>12</sub>: absorption and binding proteins. PhD Thesis, University of Manchester.
- Waters H.M., Smith C., Howarth J.E., Dawson D.W. & Delamore I.W. (1989) New enzyme immunoassay for detecting total, type I, and type II intrinsic factor antibodies. *Journal of Clinical Pathology* **42**, 307-312.